Plasma proteomic profiling and pathway analysis of normal and overconditioned dairy cows during the transition from late pregnancy to early lactation

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ABSTRACT

This study applied a quantitative proteomics approach along with bioinformatics analyses to investigate changes in the plasma proteome of normal and overconditioned dairy cows during the transition period. Fifteen weeks before their anticipated calving date, 38 multiparous Holstein cows were selected based on their current and previous body condition scores (BCS) and allocated to either a high or a normal BCS group (19 cows each). They received different diets until dry-off to reach targeted differences in BCS and back fat thickness (BFT) until dry-off. At dry-off, normal BCS cows had a BCS <3.5 (minimum, 2.75) and BFT <1.2 cm (minimum, 0.58), and the high BCS cows had a BCS >3.75 (maximum, 4.50) and BFT >1.4 cm (maximum, 2.90). The proteomics study used a subset of 5 animals from each group. These cows were selected based on their circulating concentrations of fatty acids (FA) on d 14 postpartum and β -hydroxybutyrate (BHB) on d 21 postpartum, representing the greater or the lower extreme values within their BCS group, respectively. The high BCS subset (HE-HBCS) had $4.50 < \text{BCS} < 3.75$, $\text{FA} = 1.17 \pm 0.46$ mmol/L, and $\text{BHB} = 2.15 \pm 0.42$ mmol/L (means $\pm$ SD), and the low BCS subset (LE-NBCS) had $3.50 < \text{BCS} < 2.75$, $\text{FA} = 0.51 \pm 0.28$

mmol/L, and $\text{BHB} = 0.84 \pm 0.17$ mmol/L. Plasma samples from d -49, +7, and +21 relative to parturition were used for proteome profiling by applying the quantitative tandem mass tags (TMT) approach. Non-depleted plasma samples were subjected to reduction and digestion and then labeled with TMT 10plex reagents. High-resolution liquid chromatography-tandem mass spectrometry analysis of TMT-labeled peptides was carried out, and the acquired spectra were analyzed for protein identification and quantification. In total, 254 quantifiable proteins (criteria: 2 unique peptides and 5% false discovery rate) were identified in the plasma samples. From these, 24 differentially abundant proteins (14 more abundant, 10 less abundant) were observed in the LE-NBCS cows compared with the HE-HBCS cows during the transition period. Plasma α -2-macroglobulins were more abundant in HE-HBCS versus LE-NBCS cows at d +7 and +21. Gene Ontology enrichment analyses of differentially abundant proteins revealed that the acute inflammatory response, regulation of complement activation, protein activation cascade, and regulation of humoral immune response were the most enriched terms in the LE-NBCS group compared with the HE-HBCS group. In addition, we identified 24 differentially abundant proteins (16 in the LE-NBCS group, and 8 in the HE-HBCS group) during the transition period. The complement components C1q and C5 were less abundant, while C3 and C3d were more abundant in LE-NBCS compared with HE-HBCS cows. Overall, overconditioning around calving was associated with alterations in protein pathways related to acute inflammatory response and regulation of complement and coagulation cascades in transition cows.

Key words: quantitative proteomics, body condition score, transition cows

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INTRODUCTION

Dairy cows undergo comprehensive metabolic adaptations during the transition period. The intensive mobilization of body fat depots during this time results in increased circulating concentrations of fatty acids (**FA**) and may also augment ketogenesis (Drackley, 1999). The FA can be used as energy substrates and are also used for the mammary synthesis of milk fat. However, when the capacity to use FA is exceeded, they may disrupt several metabolic and inflammatory functions (Contreras and Sordillo, 2011). The ability of dairy cows to cope with metabolic stress imposed by lactation is compromised when animals are overconditioned and mobilize body reserves excessively (Schuh et al., 2019). Progress has been made in unraveling the impact of overconditioning at calving on different blood metabolites (Mansouryar et al., 2018; Schuh et al., 2019) and circulating lipid intermediates (Rico et al., 2015) as well as metabolic pathways at the metabolome level (Ghaffari et al., 2019) in dairy cows. However, the effects of overconditioning on metabolic pathways at the protein level are still not well understood.

Recent technological developments in proteomics have led to a renewed interest in studying global protein alterations in animal research. The profiling of plasma proteomes is a powerful tool for characterizing the physiological status (Cho, 2007), which may then enable developing strategies to improve herd health management. Proteins circulating in the blood are a good source of biological information; however, plasma proteomic profiling is challenging because of the high dynamic range of protein abundances (Geyer et al., 2016). Proteomics studies performed in tissues and various body fluids of dairy cows during the transition period were reviewed by Ceciliani et al. (2018). The proteome in blood plasma has been described in context with hypocalcemia (Xia et al., 2012; Fan et al., 2017) and endometritis (Zhang et al., 2017; Miller et al., 2019) and during intramammary infection (Kim et al., 2011). Other proteomics studies have advanced understanding of the liver proteome of cows under heat stress (Skibieli et al., 2018) and the adipose tissue (**AT**) proteome of cows during the postpartum period (Takiya et al., 2019). In shotgun proteomic analysis, the depletion of high-abundance proteins in plasma is used to increase the detection of lower-abundance proteins (Echan et al., 2005; Gianazza et al., 2016; Pietrowska et al., 2019). However, depletion may lead to biases probably due to cross-reactions of the antibodies used in immunodepletion or by proteins bound to high-abundance carrier proteins such as albumin in the shotgun proteomic analysis (Bellei et al., 2011; Tu et al., 2010). We were interested in investigating potential differences in

the plasma proteomes of normal versus overconditioned cows and thus used the full proteome (i.e., without any depletion of abundant proteins).

Identification of important proteins associated with overconditioning around calving may help to elucidate the mechanisms involved in the pathophysiology of overconditioning in dairy cows. Therefore, we used an animal model of normal versus overcondition around calving based on a targeted divergence in body condition in late lactation (Schuh et al., 2019). Five cows from each group were selected based on the high or low extreme values of FA and BHB early postpartum to maximize the differences between the groups. A tandem mass tag (**TMT**)-based proteomic approach was applied to explore the alterations in the plasma proteome of cows during the periparturient period. Furthermore, the changed proteins were used as a starting point for creating a protein interaction network to elucidate the differentiated altered pathways using Gene Ontology (**GO**) enrichment analysis during the transition from late pregnancy to early lactation.

MATERIALS AND METHODS

Animals, Treatments, and Experimental Design

The field experiment was performed at the Educational and Research Centre for Animal Husbandry, Hofgut Neumuehle, Muenchweiler a.d. Alsenz, Germany. All animal experiments were performed according to the German Animal Welfare Act and approved by the local authority for animal welfare affairs (Landesuntersuchungsamt Rheinland-Pfalz [G 14-20-071], Koblenz, Germany). The study design with the performance results was previously presented in detail by Schuh et al. (2019). In brief, 15 wk before expected calving, 38 multiparous German Holstein cows (average parity 2.9 ± 0.3) were allocated to either a normal BCS ($n = 19$) or high BCS group ($n = 19$). To reach the targeted differences in BCS and backfat thickness (**BFT**) in the experimental groups, normal BCS cows were fed a low-energy ration (6.8 MJ NE_L/kg of DM) and high BCS cows were fed a ration with greater energy density (7.2 MJ NE_L/kg of DM; Supplemental Table S1; <https://doi.org/10.3168/jds.2019-17897>) during late lactation until dry-off (wk 7 antepartum, **ap**). The diets were balanced according to the recommendation of the German Society of Nutrition Physiology (GfE, 2001). Both groups received the same diets during the dry period and the subsequent lactation. At dry-off, the normal BCS cows had a BCS <3.5 (minimum value 2.75) and BFT <1.2 cm (minimum value 0.58), whereas the high BCS cows had a BCS >3.75 (maximum value 4.50) and BFT >1.4 cm (maximum value 2.90) (Figure 1A).

A subset of animals and samples from each group was created for the proteomic analyses, with selection based on maximal differences between the groups. Five cows were selected from each group based on their circulating concentrations of FA and BHB on d 14 and 21, respectively: cows with the upper extreme values in the high BCS group formed the **HE-HBCS** group ($4.50 < \text{BCS} < 3.75$, $\text{FA} = 1.17 \pm 0.46$ mmol/L, $\text{BHB} = 2.15 \pm 0.42$ mmol/L, means $\pm$ SD), whereas the cows with the lower extreme values within the normal BCS group made up the **LE-NBCS** group ($3.50 < \text{BCS} < 2.75$, $\text{FA} = 0.51 \pm 0.28$ mmol/L, $\text{BHB} = 0.84 \pm 0.17$ mmol/L; Figure 1B).

BCS, BFT, and Calculation of Energy Balance

Both BCS and BFT were continuously monitored by one person every 2 wk during the entire trial (15 wk ap to 12 wk postpartum, **pp**). The BCS was estimated on a 5-point scale with 0.25 increments (1 = emaciated, 5 = extremely fat; Edmonson et al., 1989), whereas BFT was assessed (Schröder and Staufenbiel, 2006) in the present study using ultrasonography (Agroscan L, ALR 500, 5-MHz linear-array transducer, Echo Control Medical, Angoulême, France).

Individual daily feed intake was recorded, and NE_M , net energy requirement for pregnancy, and NE_L , as well

Figure 1. Schematic diagram of the study and proteomic workflow. (A) Timeline of treatment application and data collection during the study period from 15 wk before expected calving date until 12 wk after calving. Arrows indicate blood sampling time points. (B) Changes of BCS and backfat thickness (BFT) of overconditioned (HBCS; >3.75 BCS and >1.4 cm BFT) and normal-conditioned (NBCS; <3.5 BCS and <1.2 cm BFT) cows during the experimental period. Data for BCS and BFT ($n = 19$ per group) are from Schuh et al. (2019). Asterisks indicate a difference (* $P < 0.05$; ** $P < 0.01$) between groups at a given time point. Data are presented as means $\pm$ SEM. A subset of animals and samples from the main experiment were used for this proteomics study: 5 cows from each group were selected based on their circulating concentrations of nonesterified fatty acids (NEFA) on d 14 postpartum (pp) and BHB on d 21 pp representing the greater or the lower extreme values within their BCS group, respectively. The high BCS subset (HE-HBCS) had $4.50 > \text{BCS} > 3.75$, fatty acids = 1.17 ± 0.46 mmol/L, and BHB = 2.15 ± 0.42 mmol/L (means $\pm$ SD), whereas the low BCS subset (LE-NBCS) had $3.50 > \text{BCS} > 2.75$, fatty acids = 0.51 ± 0.28 mmol/L, and BHB = 0.84 ± 0.17 mmol/L. (C) Procedure summary for tandem mass tag (TMT) quantitative proteomics and Gene Ontology (GO) analysis. Protein extracts isolated from blood samples were reduced, alkylated, and digested. Samples were labeled with the TMT reagents and mixed. Labeled samples were analyzed by high-resolution LC-MS/MS before data analysis to identify peptides and quantify reporter ion relative abundance. (D) Further statistical and GO enrichment analyses were performed on plasma proteome. FDR = false discovery rate.

as the milk energy concentrations, were calculated according to the guidelines of the GfE (2001) as follows (Figure 2):

$$\begin{aligned} \text{Net energy balance (MJ NE}_L\text{/d)} &= \text{energy intake} \\ &= (\text{MJ of NE}_L\text{/d}) - \text{NE}_M \text{ (MJ of NE}_L\text{/d)} \\ &\quad - \text{NE}_L \text{ (MJ of NE}_L\text{/d);} \end{aligned}$$

$$\text{NE}_M \text{ (MJ of NE}_L\text{/d)} = 0.293 \times \text{BW}^{0.75};$$

Maintenance and pregnancy

$$(6\text{--}4 \text{ wk ap; MJ of NE}_L\text{/d): NE}_M + 13;$$

Maintenance and pregnancy (3 wk ap until calving;

$$\text{MJ of NE}_L\text{/d): NE}_M + 18;$$

$$\begin{aligned} \text{Milk energy concentration (MJ of NE}_L\text{/kg)} &= 0.38 \\ &\times \text{milk fat (\%)} + 0.21 \times \text{milk protein (\%)} + 0.95; \end{aligned}$$

$$\begin{aligned} \text{NE}_L \text{ (MJ of NE}_L\text{/d)} &= [\text{milk energy concentration} \\ &\quad (\text{MJ of NE}_L\text{/kg)} + 0.086] \times \text{milk yield (kg/d);} \end{aligned}$$

$$\begin{aligned} \text{Energy intake} &= \text{daily DMI} \times \text{energy content} \\ &\quad \text{of the TMR (NE}_L\text{/kg of DM).} \end{aligned}$$

Blood Sampling

Blood samples were collected on d -49 , $+7$, and $+21$ relative to parturition by venipuncture from the coccygeal vein after the morning milking but before fresh feed was provided. For serum, Monovette tubes (Sartstedt AG & Co., Nümbrecht, Germany) were used; blood samples were left to coagulate for no more than 60 min at room temperature, centrifuged (10 min, $2,000 \times g$), and subsequently stored at -20°C until analysis. For obtaining plasma, EDTA tubes (Sarstedt AG & Co) were used. Samples were centrifuged (10 min, $3,500 \times g$), and the plasma was stored at -80°C until analyzed.

Figure 2. Energy balance in high-conditioned (HE-HBCS; $4.50 > \text{BCS} > 3.75$) and normal-conditioned (LE-NBCS; $3.50 > \text{BCS} > 2.75$) cows selected, respectively, based on the high [fatty acids (FA) = 1.17 ± 0.46 mmol/L; BHB = 2.15 ± 0.42 mmol/L; means $\pm$ SD] or low (FA = 0.51 ± 0.28 mmol/L; BHB = 0.84 ± 0.17 mmol/L) extreme values of serum FA on d 14 postpartum (pp) and BHB on d 21 pp. Symbols indicate a difference (* $P < 0.05$; ** $P < 0.01$) or a trend for a difference ($\dagger P < 0.1$) between the groups at a given time. Data for energy balance as presented (means $\pm$ SE) are from Schuh et al. (2019).

Protein Identification and Quantification Using the TMT Approach

The plasma samples were used for proteome profiling in a quantitative TMT-based approach. Samples were prepared at the Laboratory of Proteomics in the Faculty of Veterinary Medicine in Zagreb, and total protein quantification was performed by the bicinchoninic acid assay (Thermo Scientific, Rockford, IL). Afterward, samples were prepared for peptide labeling using TMT kits and reagents (TMT10plex Label Reagent Set, Thermo Scientific) according to the manufacturer's instructions as described in Horvatić et al. (2019). Briefly (Figure 1C), 35 µg of total protein from samples and internal standards (a pool of equal protein amounts from all samples used as a reference for normalization) was diluted to a volume of 50 µL using 0.1 M triethylammonium bicarbonate (TEAB, Thermo Scientific). Samples were reduced (20 mM dithiothreitol, Sigma Aldrich, St. Louis, MO) for 1 h at 55°C and alkylated (30 mM iodoacetamide, Sigma Aldrich) for 30 min at room temperature in the dark. Then, 300 µL of chilled (−20°C) acetone was added, and samples were allowed to precipitate overnight at −20°C. Protein pellets were collected subsequently by centrifugation (9,000 × g, 4°C), dissolved in 50 µL of 0.1 M TEAB, and digested on the filters at 37°C overnight by using 1 µL of trypsin (1 mg/mL; Promega, Madison, WI; trypsin-to-protein ratio 1:35). Peptides were labeled using the TMT 10plex Label Reagent Set prepared according to the manufacturer's procedure, and 19 µL of the appropriate TMT label was added to each sample used for the labeling reaction (60 min, room temperature), which was quenched by 5% hydroxylamine (Sigma-Aldrich). Nine TMT-modified peptide samples were combined with the internal standard (labeled with TMT *m/z* 126) into a new tube, aliquoted, dried, and stored at −20°C for further analysis.

High-resolution liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of TMT-labeled peptides was carried out using an Ultimate 3000 RSLCnano system (Dionex, Germering, Germany) coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The TMT-labeled peptides were dissolved in loading solvent (1% acetonitrile, 0.1% formic acid) and loaded onto the trap column (C18 PepMap100, 5 µm, 100 Å, 300 µm × 5 mm), desalted for 12 min at a flow rate of 15 µL/min and separated on the analytical column (PepMap RSLC C18, 50 cm × 75 µm) using a linear gradient of 5 to 45% mobile phase B (0.1% formic acid in 80% acetonitrile) over 120 min, 45% to 90% for 2 min, held at 80% for 2 min, and re-equilibrated at 5% B for 20

min at the flow rate of 300 nL/min. Mobile phase A consisted of 0.1% formic acid in water. Ionization was achieved using a nanospray Flex ion source (Thermo Fisher Scientific) containing a 10-µm-i.d. SilicaTip emitter (New Objective, Woburn, MA). The MS was operated in positive ion mode using the DDA Top8 method. Full-scan MS spectra were acquired in the range from *m/z* 350.0 to *m/z* 1800.0 with a resolution of 70,000, 110-ms injection time, automated gain control (AGC) target 1×10^6 , a ± 2.0 -Da isolation window, and the dynamic exclusion 30 s. Higher-energy collisional dissociation fragmentation was performed at step collision energy (29 and 35% normalized collisional energy) with a resolution of 17,500 and AGC target of 2×10^5 . Precursor ions with unassigned charge state as well as those with charge states of +1 and more than +7 were excluded from fragmentation.

Acquired MS/MS spectra were analyzed for protein identification and quantification using the SEQUEST algorithm implemented into Proteome Discoverer (version 2.0., ThermoFisher Scientific). Database search against *Bos taurus* FASTA files (downloaded from SwissProt database on May 11, 2016, 6,365 sequences) was performed according to the following parameters: 2 trypsin missed cleavage sites, precursor and fragment mass tolerances of 10 ppm and 0.02 Da, respectively; carbamidomethyl (C) fixed peptide modification; and oxidation (M), deamidation (N, Q), and TMT 10 plex (K, peptide N terminus) dynamic modifications. The false discovery rate (FDR) for peptide identification was calculated using the Percolator algorithm in the Proteome Discoverer workflow based on the search results against a decoy database and was set at 1% FDR. However, at least 2 unique peptides and 5% FDR were required for reporting confidently identified proteins. Protein quantification was accomplished by correlating the relative intensities of reporter ions extracted from tandem mass spectra to that of the peptides selected for MS/MS fragmentation. The internal standards were used to compare relative quantification results for each protein between the different 10-plex mixtures.

Statistical Analysis and Bioinformatics for the Proteomics Data

A repeated-measures model was fitted to energy balance data using the PROC MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC) with a first-order autoregressive covariance structure applied. The model consisted of group, parity, time, and the interaction of group and time as fixed effects and cow as a random effect. The Tukey-Kramer adjustment was applied to account for multiple comparisons. The threshold of

significance was set at $P \leq 0.05$; trends were declared at $0.05 < P \leq 0.10$. Group effect (LE-NBCS vs. HE-HBCS) and time effect in each group (d -49, d +7, and d +21 relative to calving) were considered for investigation. A protein was not considered for the analysis if one of its groups (group, time, or group $\times$ time) had more than 50% of data missing after LC-MS/MS identification and quantification. Statistical analysis was performed using R (v3.4.3) under the RStudio environment (v1.0.143). Kruskal-Wallis test with post hoc was applied to calculate P -values to determine statistical significance among both groups of cows at different time points using the R package “PMCMRplus.” Due to multiple comparisons performed, a local FDR was applied using the R package “qvalue.” Each P -value was transformed by the function $-\log_{10}(x)$. Fold change between the 2 groups was calculated by the function $\log_2 [\text{mean (Group 2)}/\text{mean (Group 1)}]$. A volcano plot was designed using the R package “plotly.” The volcano plots were generated with the “ggplot2” package.

Proteins' ID was converted into Gene ID by the platform DAVID (david-d.ncifcrf.gov/conversion.jsp) conversion tool. The GO enrichment analysis was performed using the Cytoscape (v3.6.0; Shannon et al., 2003) plugin ClueGO (v2.5.0; Bindea et al., 2009) on GO-Biological Processes. For the group effect (LE-NBCS vs. HE-HBCS), 2 clusters of proteins differentially abundant between the 2 groups were set: one cluster for overexpressed proteins and the other for proteins exhibiting lower expression. For each effect (group and time), the list of proteins was enriched for their best interactors using IntAct and Reactome databases (max 5 best interactors per protein). The 2 lists of GO overexpressed in the context of group and time effects were submitted to an analysis by REVIGO (Suppek et al., 2011) to remove redundant GO and group similar terms based on their description. The analysis was performed using the following parameters: evidence code = All, GO levels 3 to 15, the minimal number of genes = 3, the minimal percentage of genes = 3, Kappa score threshold = 0.4, and P -values corrected by Bonferroni step down. On the interactomes, original proteins are represented in a different way than the enriched proteins, and their fold change is represented with a filling color (Figures 3–5). Unmapped proteins are represented as well. Each GO term is defined by the number of genes or proteins belonging to it (enriched + original) and its associated value (in $-\log_{10}$). A map of different GO terms in different contexts has been made to visualize common ones (Figure 3B, Figure 4B, Figure 5B, and Figure 6). Node size of 1 GO term is proportional to the number of contexts in which this GO is overrepresented. Each GO family is represented by a dedicated color.

RESULTS

Plasma Proteomic Changes

In this study, 254 quantifiable proteins [151 master proteins, i.e., the top-ranked protein in a group of proteins that is identified by a set of peptides that are not included (all together) in any other protein group] were identified by a label-based quantitative proteomic approach according to the set criteria (2 unique peptides and $\leq 5\%$ FDR). A complete list of all proteins identified is provided in Supplemental Table S2 (<https://doi.org/10.3168/jds.2019-17897>).

Overconditioning around calving had effects on plasma proteins as illustrated by the volcano plots on d -49 (Figure 3A), +7 (Figure 4A), and +21 (Figure 5A). We found 24 differentially ($P < 0.05$) abundant proteins between LE-NBCS and HE-HBCS cows (Table 1). In total, 8 proteins were differentially ($P < 0.05$) abundant on d -49. Of these proteins, adiponectin, CD5L protein, immunoglobulin J chain, complement C1q, and sex hormone-binding globulin were less abundant and angiotensinogen (AGT), α -1-antiproteinase (SERPINA1), and alpha1-antichymotrypsin (SERPINA3) were more abundant in LE-NBCS compared with HE-HBCS cows. On d +7, a total of 10 differentially ($P < 0.05$) abundant proteins, including α -2-HS-glycoprotein, β -2-microglobulin, pentaxin, extracellular matrix protein 1, fibronectin, kininogen-1, complement C4 binding protein, thrombospondin-1, and one hemoglobin subunit delta were found to be more abundant and α -2-macroglobulins (A2M) were less abundant in LE-NBCS compared with HE-HBCS cows. Eight proteins were differentially ($P < 0.05$) abundant on d +21, including complement component 5 (C5), α -amylase, coagulation factor IX, albumin, and A2M, which were less abundant, and complement component 3d (C3), complement C3, and C4b-binding protein α chain (C4BPA), which were more abundant, in LE-NBCS compared with HE-HBCS cows.

Bioinformatics Analyses (Group Effect)

The changed proteins were used as a starting point for creating GO. From the GO analysis of d -49 (Figure 3B), +7 different GO between the groups (LE-NBCS vs. HE-HBCS) were defined as follows: acute inflammatory response (9 genes, $-\log_{10} P = 7.5$), acute-phase response (4 genes, $-\log_{10} P = 3.8$), regulation of IL-8 production (4 genes, $-\log_{10} P = 3.6$), regulation of macrophage-derived foam cell differentiation (3 genes, $-\log_{10} P = 3.5$), regulation of muscle cell apoptotic process (3 genes, $-\log_{10} P = 3.3$), regulation of vascular smooth muscle cell proliferation (3 genes, $-\log_{10} P =$

3.1), and negative regulation of cellular carbohydrate metabolic process (3 genes, $-\log_{10} P = 3.0$). Figure 3C shows the interactome network of identified GO terms overrepresented in the pool of abundant plasma proteins on d -49.

From the GO analysis of d +7 (Figure 4B) samples, 10 different GO between the groups (LE-NBCS vs. HE-HBCS) were defined: acute inflammatory response (16 genes, $-\log_{10} P = 14.7$), regulation of protein processing (14 genes, $-\log_{10} P = 13.9$), regulation of acute inflammatory response (13 genes, $-\log_{10} P = 13.3$), protein activation cascade (13 genes, $-\log_{10} P = 12.1$), complement activation pathway (10 genes, $-\log_{10} P = 9.7$), lymphocyte migration (6 genes, $-\log_{10} P = 4.7$), apoptotic cell clearance (5 genes, $-\log_{10} P = 5.8$), regulation of phagocytosis (5 genes, $-\log_{10} P = 4.4$), positive regulation of protein localization to plasma membrane (4 genes, $-\log_{10} P = 3.7$), and negative regulation of

blood coagulation (3 genes, $-\log_{10} P = 2.7$). Figure 4C shows the interactome network of identified GO terms overrepresented in the pool of abundant plasma proteins on d +7. Representation of GO showed that acute inflammatory response was the most affected pathway by overconditioning at dry-off and after calving in this study.

From the GO analysis of d +21 (Figure 5B) samples, 10 different GO between the groups (LE-NBCS vs. HE-HBCS) were defined as follows: protein activation cascade (14 genes, $-\log_{10} P = 17.1$), regulation of humoral immune response (12 genes, $-\log_{10} P = 15.7$), acute inflammatory response (12 genes, $-\log_{10} P = 12.4$), regulation of complement activation (10 genes, $-\log_{10} P = 12.9$), regulation of phagocytosis (5 genes, $-\log_{10} P = 5.7$), positive regulation of apoptotic cell clearance (4 genes, $-\log_{10} P = 7.9$), blood coagulation intrinsic pathway (4 genes, $-\log_{10} P = 6.5$), and positive regula-

Figure 3. (A) Volcano plot illustrating plasma proteome differences on d -49 relative to calving in high-conditioned (HE-HBCS; $4.50 > \text{BCS} > 3.75$) and normal-conditioned (LE-NBCS; $3.50 > \text{BCS} > 2.75$) cows selected, respectively, based on the high [fatty acids (FA) = 1.17 ± 0.46 mmol/L; BHB = 2.15 ± 0.42 mmol/L; means $\pm$ SD] or low (FA = 0.51 ± 0.28 mmol/L; BHB = 0.84 ± 0.17 mmol/L) extreme values of serum FA on d 14 postpartum (pp) and BHB on d 21 pp. Volcano plot of fold changes (x-axis) and their associated $\log_{10}$ transformed P -values (y-axis) for the 254 proteins analyzed by LC-MS/MS. Proteins that were different between HE-HBCS and LE-NBCS groups ($\log_{10} P > 1.3$) are shown in green, and proteins that did not differ ($\log_{10} P < 1.3$) are depicted in red. (B) Gene ontology (GO) enrichment analysis of differentially expressed plasma proteins on d -49 relative to calving. The GO terms are ordered by the number of genes or proteins of the study associated with them according to the GO database (first y-axis). The $-\log_{10} P$ -value for each GO term is represented on the second y-axis. (C) Interactome network of identified GO terms overrepresented in the pool of abundant plasma proteins on d -49 relative to calving.

tion of blood coagulation (3 genes, $-\log_{10} P = 3.8$). Figure 5C shows the interactome network of identified GO terms overrepresented in the pool of abundant plasma proteins on d +21. Representation of GO terms showed that regulation of humoral immune response was the pathway most affected by overconditioning on d +21 in this study.

Bioinformatics Analysis (Time Effect)

From the analysis of GO annotation, we classified enriched gene functional categories for LE-NBCS and HE-HBCS during the transition period as shown in Figure 6. The bar graph represents the number of proteins belonging to the corresponding GO, and the line graph is taken from $-\log_{10}$ of the p-value. Twenty-five different GO were defined in LE-NBCS (Figure 6A)

and 15 different GO were defined in HE-HBCS (Figure 6B) during the transition period.

Among all proteins, haptoglobin (**Hp**) and adiponectin were measured at more time points via in-house developed ELISA. The serum concentrations of Hp and adiponectin ($n = 5$ per group) during the transition from late pregnancy to early lactation are presented in Supplemental Figure S1 (<https://doi.org/10.3168/jds.2019-17897>). The serum concentrations of Hp concentrations were elevated in the first week after calving ($P < 0.01$) as compared with other time points but did not differ between the groups (Supplemental Figure S1A). As indicated by the time by group interaction for adiponectin (Supplemental Figure S1B), the levels of adiponectin in HE-HBCS and LE-NBCS cows were not parallel but crossed: the lower values were mostly observed in LE-NBCS cows antepartum but the values

Figure 4. (A) Volcano plot illustrating plasma proteome differences on d +7 relative to calving in high-conditioned (HE-HBCS; $4.50 > \text{BCS} > 3.75$) and normal-conditioned (LE-NBCS; $3.50 > \text{BCS} > 2.75$) cows selected, respectively, based on the high [fatty acids (FA) = 1.17 ± 0.46 mmol/L; BHB = 2.15 ± 0.42 mmol/L; means $\pm$ SD] or low (FA = 0.51 ± 0.28 mmol/L; BHB = 0.84 ± 0.17 mmol/L) extreme values of serum FA on d 14 postpartum (pp) and BHB on d 21 pp. Volcano plot of fold changes (x-axis) and their associated $\log_{10}$ transformed P -values (y-axis) for the 254 proteins analyzed by LC-MS/MS. Proteins that were different between HE-HBCS and LE-NBCS groups ($\log_{10} P > 1.3$) are shown in green, and proteins that did not differ ($\log_{10} P < 1.3$) are depicted in red. (B) Gene Ontology (GO) enrichment analysis of differentially expressed plasma proteins on d +7 relative to calving. The GO terms are ordered by the number of genes or proteins of the study associated with them according to the GO database (first y-axis). The $-\log_{10} P$ -value for each GO term is represented on the second y-axis. (C) Interactome network of identified GO terms overrepresented in the pool of abundant plasma proteins on d 7 relative to calving.

Figure 5. (A) Volcano plot illustrating plasma proteome differences on d +21 relative to calving in high-conditioned (HE-HBCS; $4.50 > \text{BCS} > 3.75$) and normal-conditioned (LE-NBCS; $3.50 > \text{BCS} > 2.75$) cows selected, respectively, based on the high [fatty acids (FA) = 1.17 ± 0.46 mmol/L; BHB = 2.15 ± 0.42 mmol/L; means $\pm$ SD] or low (FA = 0.51 ± 0.28 mmol/L; BHB = 0.84 ± 0.17 mmol/L) extreme values of serum FA on d 14 postpartum (pp) and BHB on d 21 pp. Volcano plot of fold changes (x-axis) and their associated $\log_{10}$ transformed P -values (y-axis) for the 254 proteins analyzed by LC-MS/MS. Proteins that were different between the HE-HBCS and LE-NBCS groups ($\log_{10} P > 1.3$) are shown with green symbols, and proteins that did not differ ($\log_{10} P < 1.3$) have red symbols. (B) Gene Ontology (GO) enrichment analysis of differentially expressed plasma proteins on d +21 relative to calving. The GO terms are ordered by the number of genes or proteins of the study associated with them according to the GO database (first y-axis). The $-\log_{10} P$ -value for each GO term is represented on the second y-axis. (C) Interactome network of identified GO terms overrepresented in the pool of abundant plasma proteins on d +21 relative to calving.

of the LE-NBCS cows exceeded those of the HE-HBCS cows in early lactation.

DISCUSSION

The aim of the current study was to investigate differences in the plasma proteome in normal BCS and high BCS cows that were selected according to extreme blood parameters (nonesterified FA and BHB) during the transition from late gestation to early lactation. For this purpose, a TMT-based quantitative proteomics approach was performed to identify and quantify proteins. In plasma, several high-abundance proteins are dominant and can mask the detection of the very low abundance proteins (Echan et al., 2005). Therefore, depletion strategies are commonly used to enhance the detection of lower abundance proteins in both shotgun and targeted proteomic analyses (Echan et al., 2005;

Gianazza et al., 2016; Pietrowska et al., 2019). However, immunodepletion in the shotgun proteomic analysis may lead to biases because of the removal of some nontargeted proteins that may be of potential interest along with the targeted proteins such as albumin (Tu et al., 2010). With this in mind, we decided to omit any steps to deplete high-abundance plasma proteins in this study.

In our protein profiling experiment, we identified 24 differentially abundant proteins (16 proteins in LE-NBCS, and 8 proteins in HE-HBCS) during the transition period. Plasma lipoprotein particle remodeling and regulation of lipase activity were identified as altered pathways during the transition period in both groups by GO enrichment analysis. Dairy cows experiencing high mobilization of body fat depots in early lactation due to a negative energy balance have an increased release of FA and ketone bodies into the

circulation (Dale et al., 1979; Weber et al., 2013). Thus, an imbalance between lipomobilization and hepatic lipid disposal (accumulation of triglycerides and low rates of hepatic very low density lipoprotein [VLDL] synthesis and secretion inhibiting lipid export) leads to fatty liver and ketosis in early lactation (Pullen et

al., 1990; Drackley, 1999). As described in the review by Gruffat et al. (1996), the synthesis and secretion of VLDL in the liver are complex processes. The possible mechanisms for reduced VLDL secretion in fatty liver cows could be (1) reduced endoplasmic reticulum to Golgi apparatus transport of VLDL, (2) alterations in

Figure 6. Time-affected proteins during the transition from late gestation to early lactation in (A) normal-conditioned (LE-NBCS; $3.50 > \text{BCS} > 2.75$) and (B) high-conditioned (HE-HBCS; $4.50 > \text{BCS} > 3.75$) cows selected, respectively, based on the high [fatty acids (FA) = 1.17 ± 0.46 mmol/L; BHB = 2.15 ± 0.42 mmol/L; means $\pm$ SD] or low (FA = 0.51 ± 0.28 mmol/L; BHB = 0.84 ± 0.17 mmol/L) extreme values of serum FA on d 14 postpartum (pp) and BHB on d 21 pp. Bar plot of the mean of 8 proteins (for LE-NBCS cows) and 16 proteins (for HE-HBCS cows) differentially abundant for the different time points (d -49, d +7, and d +21 relative to calving).

Table 1. Proteins with differential abundance identified using the tandem mass tags approach in overconditioned (HE-HBCS) and normal-conditioned (LE-NBCS) cows at different time points (d -49, d +7, and d +21 relative to calving)

Group and accession no.	Gene symbol	Protein name	Wilcoxon <i>P</i> -value			Fold change		
			d -49/d 7	d -49/d 21	d 7/d 21	d -49/d 7	d -49/d 21	d 7/d 21
HE-HBCS								
Q3Y5Z3	<i>ADIPOQ</i>	Adiponectin (<i>Bos taurus</i>)	0.12	0.06	0.71	-0.44	0.58	-0.14
Q3SZH5	<i>AGT</i>	Angiotensinogen (<i>Bos taurus</i>)	0.16	**	0.16	0.21	-0.21	0.00
P19035	<i>APOC3</i>	Apolipoprotein C-III (<i>Bos taurus</i>)	***	***	0.82	0.43	0.00	-0.42
Q2KIH2	<i>APOF</i>	ApoN protein (<i>Bos taurus</i>)	0.14	0.10	0.82	0.22	0.11	-0.33
Q2UVX4	<i>C3</i>	Complement C3 (<i>Bos taurus</i>)	0.87	0.09	0.07	-0.22	0.26	-0.03
Q32PA1	<i>CD59</i>	CD59 molecule, complement regulatory protein (<i>Bos taurus</i>)	*	0.42	0.19	-0.04	0.32	-0.28
P23805	<i>CGN1</i>	Conglutinin (<i>Bos taurus</i>)	**	*	0.57	-0.77	0.65	0.12
P42916	<i>CL43</i>	Collectin-43 (<i>Bos taurus</i>)	**	**	**	-1.01	0.99	0.02
A6QP30	<i>CPN2</i>	CPN2 protein (<i>Bos taurus</i>)	0.07	0.56	0.23	0.31	-0.13	-0.18
P00735	<i>F2</i>	Prothrombin (<i>Bos taurus</i>)	0.93	**	**	0.22	-0.26	0.04
P02672	<i>FGA</i>	Fibrinogen alpha chain (<i>Bos taurus</i>)	***	***	0.73	0.21	-0.47	0.26
Q2KLI3	<i>HAVCR1</i>	Hepatitis A virus cellular receptor 1 (<i>Bos taurus</i>)	***	***	***	0.43	-0.02	-0.41
G3N1Y3	<i>HBD</i>	Hemoglobin subunit delta	0.37	*	0.22	0.38	-0.50	0.12
P56652	<i>ITIH3</i>	Inter-alpha-trypsin inhibitor heavy chain (<i>Bos taurus</i>)	0.12	0.06	0.71	0.26	-0.48	0.22
P41361	<i>SERPINC1</i>	Antithrombin-III (<i>Bos taurus</i>)	***	***	0.91	-0.17	0.33	-0.16
G3X6N3	<i>TF</i>	Serotransferrin (<i>Bos taurus</i>)	**	***	0.13	-0.19	0.16	0.04
LE-NBCS								
AHSG	<i>P12763</i>	Alpha-2-HS-glycoprotein (<i>Bos taurus</i>)	0.11	0.07	0.82	-0.09	0.17	-0.08
APOB	<i>E1BNR0</i>	Apolipoprotein B (<i>Bos taurus</i>)	0.37	0.15	*	-0.31	0.38	-0.08
APOC3	<i>P19035</i>	Apolipoprotein C-III (<i>Bos taurus</i>)	*	0.67	**	0.21	0.54	-0.75
SFTPD	<i>P42916</i>	Collectin-43 (<i>Bos taurus</i>)	**	**	0.78	-1.46	1.18	0.27
HP	<i>Q9MYV8</i>	Haptoglobin (<i>Bos taurus</i>)	*	***	***	-1.22	-2.84	4.06
KLKB1	<i>Q2KJ63</i>	Plasma kallikrein (<i>Bos taurus</i>)	***	***	0.13	-0.12	-0.11	0.23
SERPINA3	<i>Q9TTE1</i>	Serpin A3-1 (<i>Bos taurus</i>)	***	0.75	**	0.25	-0.34	0.10
TF	<i>E1BI82</i>	Uncharacterized protein (<i>Bos taurus</i>)	***	***	*	-0.19	0.32	-0.13

Asterisks indicate a significant difference (**P* < 0.05; ***P* = 0.01; ****P* < 0.01) from corresponding value.

apoB100 glycosylation, (3) reduced formation of secretory vesicles, and (4) reduced migration of secretory vesicles from Golgi apparatus to cell membrane (Grufat et al., 1996). Marcos et al. (1990) noted that the mRNA abundance of apolipoprotein B (**ApoB**) in the liver of cows with fatty liver was lower than in healthy cows and suggested that hepatic synthesis of apolipoprotein is impeded in dairy cows with fatty liver. Furthermore, Uchida et al. (1992) reported that dairy cows with fatty liver had a lower plasma concentration of ApoB100 compared with healthy cows.

Here, apolipoprotein C-III (**ApoCIII**) related to lipid metabolism was shown to be decreased in early lactation. Apolipoprotein C-III is primarily synthesized by the liver and plays several roles in lipid metabolism (van der Ham et al., 2009). Apolipoprotein CI (**ApoCI**) and CIII, present in VLDL and high-density lipoprotein, are mainly involved in inhibition of lipoprotein lipase activity (Jong et al., 1999). Previously, van der Ham et al. (2009) concluded that high concentrations of plasma ApoCI and ApoCIII are associated with elevated triglycerides and reduced visceral fat mass in humans with metabolic syndrome. In addition, ApoCIII is a multifaceted protein that is not only involved in triglyceride metabolism but also participates in the regulation of inflammation (Luo and Peng, 2016). In a human study, Wu et al. (2017) reported that plasma ApoCIII levels were positively associated with inflammatory markers (i.e., high sensitivity C-reactive protein). Apolipoprotein B100 is one of the main structural proteins of VLDL and is essential for stabilizing the VLDL particles (Bernabucci et al., 2004). In this study, downregulation of plasma ApoB and ApoCIII in HE-HBCS cows in early lactation suggests an impaired assembly of VLDL that may contribute to the hepatic triglyceride accumulation. Unfortunately, hepatic triglyceride levels were not measured in this study.

The transition period in dairy cattle is characterized by an inflammatory status with marked changes in the efficiency of the bovine immune system, which coincides with metabolic abnormalities (Sordillo and Raphael, 2013; Bradford et al., 2015). In the current study, the protein activation cascade related to inflammation, the immune response, and the acute phase response were the most affected pathways in both groups during the transition period. The inflammation process, which includes the acute-phase response, is mediated by cytokines derived from the activated cells of the immune system (Trey and Kushner, 1995) but also from adipocytes (Maurizi et al., 2018). The release of AT-derived proinflammatory cytokines can suppress the intracellular signaling of insulin and promote insulin resistance in peripheral tissues in dairy cows (Sordillo and Raphael, 2013). In addition, inflammation is as-

sociated with greater cellular respiration and mitochondrial uncoupling protein gene expression (Puigserver et al., 2001). Cows with higher BCS at calving were previously demonstrated to exhibit greater concentrations of systemic indicators of inflammation (Akbar et al., 2015). Overconditioned high-producing dairy cows inevitably go into a more severe negative energy balance postpartum than cows that have a normal appetite (Rukkwamsuk et al., 1999). The energy balance in the high BCS cows in our study was also more negative than in normal BCS cows, pointing toward compromised immunity and an elevated inflammatory response (Sordillo and Raphael, 2013; Wankhade et al., 2017). Beside BCS per se, the peripartur loss of body condition may increase pro-inflammatory cytokines such as IL-6 and tumor necrosis factor α in dairy cows (Kasimanickam et al., 2013). However, further studies are still needed to elucidate the alterations in protein activation cascade pathways in the transition period.

The biological functions of the proteins with highly different protein level changes between LE-NBCS and HE-HBCS cows were explored using GO enrichment analyses in this study. Gene Ontology terms were used to gain a deeper understanding of the biological significance and interactions between the differentially abundant key proteins in LE-NBCS compared with HE-HBCS cows. In the current study, GO enrichment analysis revealed that the complement system and the coagulation cascade were associated with overconditioning around calving. Recent proteomic analysis in subcutaneous AT also revealed a greater abundance of complement and inflammatory proteins in dairy cows during the postpartum period (Takiya et al., 2019). However, the actual mechanism involved in the altered complement system and coagulation cascades by overconditioning remains unclear, particularly for transition cows.

In regard to the values of complement component in the current study, complement components C1q (subcomponent subunit B), C5, and coagulation factor IX proteins were more abundant but complement components C3 and C3d were less abundant in the plasma of HE-HBCS compared with LE-NBCS cows. The complement system is a complex enzymatic cascade (consisting of more than 30 proteins in blood plasma) that function in stepwise activation of several proteases, which are produced by the liver, AT, leukocytes, and vascular cells (Vlaicu et al., 2016; Lubbers et al., 2017). Plasma C3 is important to the complement activation and is synthesized mainly in hepatocytes, but it can also be secreted by adipocytes, macrophages, and endothelial cells (Hertle et al., 2014). The complement component C1q primarily binds to antigen-bound antibody molecules and provides a recognition and

activation signal leading to activation of the classical complement pathway (Tran et al., 2017). In obesity, elevated plasma levels of free FA and insulin can activate the complement system, resulting in the production of elevated levels of complement fragments C3a and C5a (Phieler et al., 2013). Through the binding of C3a and C5a to their respective receptors (C3aR and C5aR), complement components could stimulate the formation of triglycerides by inhibiting lipolysis, enhancing glucose and free FA uptake, and thus indirectly reduce free FA release (Phieler et al., 2013). Generally, the complement system and coagulation cascades closely interact with each other (Markiewski et al., 2007; Amara et al., 2010). To the best of our knowledge, evaluations of the protein expression of the complement system of overconditioned dairy cows are largely unexplored. However, to elucidate the role of complement factors (C1q, C3, and C5), changes in the complement system functioning receptors (C3a and C5a), and its association with overconditioning around calving in dairy cows warrant further studies.

In the present study, plasma A2M was more abundant in HE-HBCS relative to LE-NBCS cows in early lactation. Alpha 2-macroglobulin is a multifunctional protein that can inhibit a broad range of proteases, including trypsin, thrombin, and collagenase (Wyatt et al., 2013), and can also regulate the binding of transferrin to its surface receptor (Rehman et al., 2013). Recent studies in transition dairy cows have identified A2M in circulating exosomes as unique to cows with a high risk of metabolic dysfunction (Crookenden et al., 2016). Furthermore, SERPINA1, SERPINA3, and AGT were found to be differentially represented between the groups and were less abundant in HE-HBCS compared with LE-NBCS cows. Alpha-1-antiproteinase (SERPINA1) is an acute-phase protein (**APP**), which is predominantly synthesized in the liver and to a lesser extent in macrophages, monocytes, and platelets (Nachman and Harpel, 1976; Perlmutter et al., 1985). Alpha-1-antichymotrypsin (SERPINA3), plays a role in the inflammatory response in cattle (Herrera-Mendez et al., 2006). Multiple isoforms of SERPINA3 (serpinA3-1 to serpinA3-8) were demonstrated in the skeletal muscle of cattle (Pr-Brissaud et al., 2015) and found to be released during an inflammatory response (Hunt and Tuder, 2012). Angiotensinogen is the precursor of angiotensin, a peptide hormone that causes vasoconstriction and an increase in blood pressure, but it has also been proposed as an APP. It is mainly synthesized in the liver and is also expressed abundantly in AT (Klett et al., 1993). In line with that, a more pronounced activation of the immune system (greater concentrations of proinflammatory cytokines, myeloperoxidase, and oxidants in plasma) was recently

reported in ketotic cows compared with healthy cows before calving (Mezzetti et al., 2019).

Haptoglobin is a prominent APP in cattle; it was one of the most differently abundant proteins in LE-NBCS cows during the transition from late gestation through early lactation. For that reason, in this study, we measured Hp via ELISA at different time points. The ELISA data were in agreement with the proteomic analysis, and a similar pattern of Hp concentrations was observed during the transition period. It is well known that parturition is related to inflammatory processes and positive APP such as Hp are elevated in the circulation around calving (Hachenberg et al., 2007; Bradford et al., 2015), which is in line with our findings. Bertoni et al. (2008) studied the relationship between inflammation and liver function in dairy cows and reported that cows with a lower liver activity index had greater inflammation with high Hp concentrations compared with cows with a higher liver activity index in early lactation.

The ELISA data for adiponectin were in agreement with the proteomic analysis in the current study as indicated by the time by group interaction, with lower values of adiponectin being observed in LE-NBCS than in HE-HBCS cows during the antepartum period. Adiponectin, a protein exclusively produced by adipocytes, is known for its insulin-sensitizing effects on the liver, skeletal muscle, and AT (Sauerwein and Hussler, 2016). Previous studies demonstrated that circulating concentrations of adiponectin in dairy cows reach the lowest values in early lactation (Sauerwein and Hussler, 2016), and its reduction could contribute to the insulin resistance of transition cows (Krumm et al., 2017). We therefore also measured adiponectin at different time points in this study. Overall, we found evidence of broad differences in plasma proteome in cows of high compared with normal BCS around the transition period.

CONCLUSIONS

The proteomic profiles of plasma differed between HE-HBCS and LE-NBCS cows during the transition period. Twenty-four differentially abundant proteins (14 more abundant; 10 less abundant) were discovered between the 2 groups during the transition period. Gene Ontology analyses of these proteins revealed that acute inflammatory response, regulation of complement, and coagulation cascades, as well as regulation of humoral immune response, were the most enriched terms in the LE-NBCS group compared with HE-HBCS group. We found that the plasma proteome levels were strongly affected by time, undergoing longitudinal changes, in which the protein activation cascades related to inflam-

mation, the immune response as well as the acute phase response, were the most affected pathways in all cows. These data point to the many changes occurring in the immune system components of periparturient dairy cows. Such information is important for improving the differentiation of normal versus abnormal antepartum or postpartum adaptation that may help to develop suitable intervention for reducing health disturbances and metabolic stress in the following lactation. Our results present a first step toward understanding the complexity of longitudinal and individual variation in the plasma proteome of overconditioned cows.

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